Failure of Viable Nonculturable Campylobacter jejuni to Colonize the Cecum of Newly Hatched Leghorn Chicks

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Research Note—

Failure of Viable Nonculturable Campylobacter jejuni to Colonize the Cecum of Newly Hatched Leghorn Chicks

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SUMMARY. *Campylobacter jejuni* cells entered the viable but nonculturable (VBNC) state upon suspension in sterile water. Cell viability was determined with tetrazolium violet. VBNC cells suspended in water for 7, 10, or 14 days were given, by gavage, to day-of-hatch leghorn chickens. The ceca of control and challenged birds were examined for the presence of campylobacteria by conventional microbiological methods at 1 wk and 2 wk after challenge inoculation and by polymerase chain reaction methods at 1 wk after challenge. We did not find culturable *Campylobacter* cells in the ceca. Neither was *Campylobacter* DNA found in cecal samples. Therefore, VBNC cells did not revert to the culturable colonizing form, nor did VBNC cells persist within the cecal environment.

RESUMEN. *Nota de Investigación*—El Campylobacter jejuni viable pero no cultivable no coloniza el ciego de pollitos leghorn recién nacidos.

El *Campylobacter jejuni* cuando se suspende en agua estéril, pasa a un estado viable pero no cultivable. La viabilidad del *Campylobacter* fue determinada con tetrazolium violeta. Las células de *Campylobacter* viables no cultivables suspendidas en agua durante 7, 10 o 14 días fueron administradas a pollitos recién nacidos por medio de sonda directamente al buche. Una y 2 semanas después de la inoculación, se examinó el ciego de los pollitos controles y de los desafiados para la presencia de *Campylobacter* utilizando métodos microbiológicos normales, lo mismo que la reacción en cadena por la polimerasa realizada una semana después del desafio. No se cultivó el *Campylobacter* a partir del ciego, como tampoco se detectó su DNA en las muestras del ciego. Por lo tanto, las células viables no cultivables no revirtieron a su forma de colonia cultivable ni persistieron en el ambiente del ciego.

Key words: *Campylobacter,* viable nonculturable

Abbreviations: INT = 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; PCR = polymerase chain reaction; ro-water = sterile water purified by reverse osmosis; VBNC = viable but nonculturable

Bacteria in the genus *Campylobacter* can be found in either of two morphologic forms, spiral or coccoid (1). *Campylobacter* cells in actively growing culture are typically elongated spiral–slightly curved rods with a single polar flagellum. When the spiral-shaped cells grow on agar surfaces for more than 48 hr, or when the cells are suspended in water or saline, they take on the coccoid shape. The cells round and become much smaller than the spiral forms, and the flagellum eventually disappears. Excellent scanning electron micrographs of both morphologic forms and extensive reviews of the relevant bacterial physiology underlying the mor-

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phologic variation have been published (6,12). The nature of these coccoid cells is unclear, and some authors consider them degenerative forms destined to die (1,5,12).

Campylobacteria also exist in either of two physiologic forms, "culturable," and "viable but nonculturable" (VBNC) (8,11). There is a somewhat limited body of scientific literature with respect to which morphologic form is VBNC. Lazaro *et al.* (6) have suggested that the physiologic change to VBNC is related to the morphologic transition to coccoid form but is a different phenomenon from the morphologic transition. Additionally, Lazaro *et al.* (6) have speculated that a spiral VBNC form exists.

Since the first description of the VBNC form of *Campylobacter,* there has been speculation concerning the role, if any, of VBNC cells in survival within aquatic environments (8). This issue is relevant to understanding how commercial flocks become contaminated with campylobacteria. Stern *et al.* (10) suggested that cells surviving in water, in VBNC form, are capable of colonizing chickens. Others have published data that support the original observation by Stern *et al.* and further suggest that VBNC cells are important in *Campylobacter* epidemiology (2,3). Yet, others hold an opposing view, that the VBNC form does not colonize chickens and is not significant to the spread of campylobacteria into broiler flocks (7,14).

Because there is uncertainty about the ability of VBNC cells to colonize chickens, we conducted the following experiments. The objective was to determine if VBNC cells formed in an aqueous environment and inoculated into day-of-hatch chicks could survive, recover, and colonize the chicks.

MATERIALS AND METHODS

Animals. Day-of-hatch leghorn chickens (HyLine W-36®) were obtained from a commercial hatchery (Hy-Line International, Bryan, TX) and placed in electrically heated commercial brooder batteries, 10 chicks per cage. Feed was heat sterilized at 65 C in an oven. Chlorinated municipal drinking water was provided in open troughs. Chicks were provided water and a balanced unmedicated corn-soybean ration *ad libitum.* An Institutional Animal Care and Use Committee reviewed and approved husbandry and experimental procedures.

VBNC. A single *Campylobacter jejuni* strain, designated in our laboratory as *C. jejuni* 91, was used for each experiment. This *C. jejuni* 91 was originally isolated from bovine fecal matter and subsequently found to be a capable colonizer of the ceca of day-of-hatch chicks (unpubl. data). Four campy-cefex agar plates (9) were heavily seeded with this organism, in a manner that did not quite lead to confluent growth, and incubated for 48 hr at 42 C in a microaerobic environment consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen. These plates were then washed thoroughly with 5 ml of sterile water that had been purified by reverse osmosis (ro-water). The resultant bacterial suspension was washed once by centrifugation and the pellets were resuspended in 45 ml of sterile ro-water. Three different batches were prepared, and each suspension was left undisturbed at room temperature for either 7, 10, or 14 days, as necessary to accommodate the experimental design. At the end of each standing period, 10-µl aliquots were applied to campy-cefex agar, to blood agar, and into *Campylobacter* enrichment broth (Lab M, Bury, England) without antibiotics. This procedure was done to determine that the cells in the suspension were nonculturable.

Assessment of viability. Two milliliters of the above mentioned cell suspensions was added to a 15-ml conical centrifuge tube containing 5 ml sterile tryptic soy broth with dextrose (Difco, Detroit, MI). Tetrazolium violet (Sigma, St. Louis, MO) was added, 0.2 ml of a tetrazolium violet–saturated water solution, and the mixture was placed in a 42 C incubator until there was a distinct color change to the mixture. The mixture was incubated for up to 1 hr, the suspension was spun in a centrifuge to sediment the cells, and appearance of a deep-violet cell pellet was taken as evidence that tetrazolium violet had been reduced after being taken up by the cells.

Cell counts and microscopy. VBNC cell suspensions were fixed for examination by scanning electron microscopy by the addition of 1 ml of cells to an equal volume of 2% glutaraldehyde in 100 mM phosphate buffer. Cells, after glutaraldehyde fixation, were washed twice in phosphate buffer, then placed onto poly-L-lysine–coated coverslips. After attachment, coverslips were again placed in 2% glutaraldehyde. After postfixation in osmium tetroxide, cells were critical point dried, coated with gold, and examined with a scanning transmission electron microscope. With dilutions of fixed cells, cell populations were estimated with the use of a Petroff–Hauser bacterial cell counting chamber and phase light microscopy. Samples of fixed and unfixed cells were examined by phase light microscopy (both wet and dry mount). Additionally, unfixed cell suspensions were air dried on glass slides, Gram stained, and examined by bright field microscopy.

Retention of identifying antigens. A latex agglutination test, INDX®-Campy (jcl)™ (Integrated Diagnostics, Baltimore, MD), was run on the 14-day VBNC cell suspension to ascertain whether the VBNC cells retained their identifying antigenic characteristics.

The manufacturer's instructions were followed except that 20 µl of the VBNC cell culture was added to the reaction mixture instead of picking colonies from culture plates.

***In vivo* challenge studies.** *In vivo* challenge inoculation studies were conducted with each of the three VBNC cell preparations, that is, with cell suspensions of 7, 10, and 14 days duration, respectively. Ten day-of-hatch chicks were given sterile water by gavage (control group), whereas another group of 10 chicks was given 0.75 ml of the VBNC cell suspension, also by gavage (treated group). One week after treatment, five chicks from each group were killed by cervical dislocation, and cecal contents were collected. The cecal material was serially diluted and plated on campy-cefex agar plates. These plates were placed in an incubator at 42 C with an atmosphere consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen. The plates were examined for the presence of *Campylobacter* colonies after approximately 40–48 hr of incubation. Additionally, cecal material from each of the inoculated chicks and one of the control chicks was examined by polymerase chain reaction (PCR) methodology for the presence of DNA sequences indicative of the presence of *C. jejuni* within the cecal matter. The above microbiological analysis was repeated with the remainder of the chicks at 2 wk after challenge inoculation, but PCR was not repeated at this second point in time.

PCR. The presence or absence of *C. jejuni* in cecal contents was also determined by PCR. Samples were placed in a boiling water bath for 15 min followed by centrifugation for 5 min at 14,000 × *g*. Portions (2 µl) of the supernatants were added to PCR reaction mixture (RedTaq™ ReadyMix™ PCR Reaction Mix; Sigma) containing PCR primers (4) (Integrated DNA Technologies, Inc., Coralville, IA). Amplification was run (PTC-200 Peltier Thermal Cycler; MJ Research, Inc., Waltham, MA) with the following parameters: 1) denaturation at 94 C for 3 min for 1 cycle; 2) denaturation at 94 C for 30 sec; 3) annealing at 57 C for 30 sec; 4) extension at 72 C for 1 min; 5) repeat steps 2 and 3 for 29 cycles; 6) extension at 72 C for 5 minutes for 1 cycle; and 7) hold at 4 C. Amplicons were examined on 1.2% agarose E-Gel (Invitrogen, Carlsbad, CA).

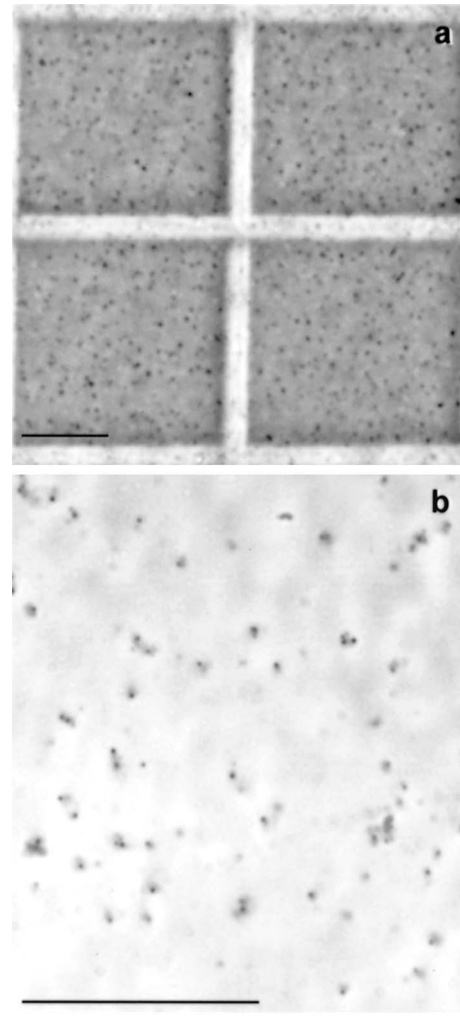

Fig. 1. Phase contrast micrographs of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)-stained bacteria after 14 days of incubation in sterile ro-water. a) Photomicrograph of an undiluted sample of the VBNC cell suspension in a Petroff–Hauser bacterial counting chamber, representing the bacterial concentration used as inoculum for day-of-hatch chicks. b) Higher magnification of INT-stained bacteria showing loss of typical spiral morphology after 14 days of incubation in H_2O. Bars = 20 µm.

RESULTS

Assessment of viability. *Campylobacter jejuni* 91 cells readily entered the viable but nonculturable state when suspended in ro-water. The nonculturable state was established by an observed failure of the cells to grow on campy-cefex agar plates, blood agar plates, or in *Campylobacter* enrichment broth. The viable nature of the cell suspensions was established by a rapid reduction of tetrazolium violet, with a concomitant readily visible color change that was noticeable within a few seconds or minutes after adding the VBNC cell suspension to the trypic soy broth–tetrazolium violet solution. In order to confirm that tetrazolium

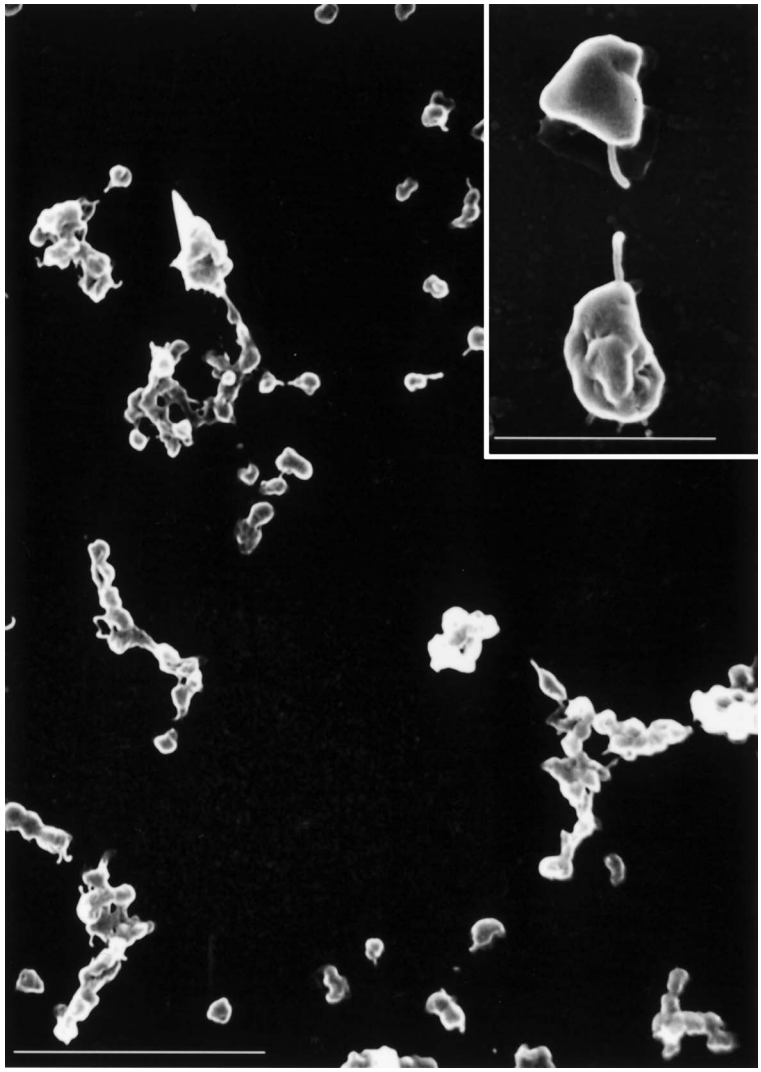

Fig. 2. Scanning electron micrographs of bacteria after 14 days of incubation in sterile ro-water. Bacteria are rounded and do not exhibit the typical spiral morphology of normal *Campylobacter*. Inset shows detail of bacteria with attached flagella. Bars = 25 μm; inset: 1 μm.

violet, which is colorless in solution, had been taken up intracellularly by the VBNC cells and reduced, we centrifuged the mixture and observed that all of the color was present in the cell pellet. The cell pellets were a deep blue-violet color. Both the depth of the color and the rapidity with which the color change occurred demonstrated that the suspended cells were in the VBNC stage and capable of rapid uptake and reduction of the tetrazolium salt.

Cell counts and microscopy. Cell counts obtained with the Petroff–Hauser bacterial cell counting chamber revealed that each VBNC suspension had approximately 5×10^9 VBNC cells/ml. The VBNC cells appeared to be very small, gram negative, and coccoid in shape when viewed by bright phase field light microscopy. The predominant coccoid cell morphology was confirmed by light and scanning electron microscopy (Figs. 1, 2).

Retention of identifying antigens. VBNC cells retained their ability to react with antibodies directed against the cell surface. A very strong and distinct agglutination reaction was observed when

these cells were used in the INDX®-Campy (jcl)™ latex slide agglutination test.

***In vivo* challenge studies.** We were unable to recover campylobacteria from our untreated control chicks or from VBNC-challenged chicks on days 7 and 14 after challenge. Therefore, none of the three VBNC cell suspensions contained cells capable of reverting from the VBNC state to a culturable form that was able to stably colonize ceca of newly hatched chicks.

PCR. Analysis by PCR of cecal material collected 1 wk after challenge inoculation with VBNC cells failed to detect DNA indicative of the presence of campylobacteria. Similarly, no *Campylobacter* DNA was detected in cecal material from control chicks. Therefore, VBNC cells did not persist or proliferate in the nonculturable form within the cecal environment.

DISCUSSION

Transformation from a culturable spiral morphology to a viable nonculturable coccoid cell is one of the most intriguing aspects of *Campylobacter* cell physiology. The cells somehow manage to maintain themselves against a steep osmotic gradient with what must be a bare-bones minimal amount of metabolism because their environment is lacking in nutrients. Despite the harsh conditions, remarkably, VBNC coccoid cells retain an ability to rapidly take up tetrazolium salts and reduce these through oxidative metabolism. The cells retain the surface antigenic structures used to identify them as *Campylobacter*, and Gram stain properties are also retained. We found that VBNC cells are difficult to counterstain with safranin, as are the spiral culturable cell forms. These characteristics along with scanning electron micrographs showing that the cells are intact, notwithstanding the extreme hypotonicity of the suspension in which the cells find themselves, are evidence of a very unusual physiologic state.

Our primary purpose for conducting these experiments was to assess the ability of VBNC cells to revert, *in vivo,* to the culturable, colonizing form because it has been proposed that the ability to enter the VBNC state is part of a mechanism by which campylobacteria survive in aqueous environments and ultimately colonize birds (8,10). Our results demonstrated an inability of VBNC cells both to revert and to colonize the cecum. Further, we showed that *Campylobacter* DNA was not present in the cecal contents of challenged chicks, indicating that perhaps the VBNC cells lysed and released their DNA at a point in the digestive system where digestive enzymes may have been active. These results indicate that hypotheses proposing that latent VBNC cells are present in the cecum prior to reversion back to the spiral form are probably wrong. Our results are very strong evidence that the VBNC cells of our *C. jejuni* strain lack the ability to revert and are most probably degenerative cells. Our results are consistent with reports by Medema *et al.* (7) and van de Giessen *et al.* (14) but are not consistent with those of Stern *et al.* (10) and Cappelier *et al.* (2).

It is certainly possible that, in nature, VBNC cells are not subjected to the extreme hypotonicity as in this experiment and are presented with more dissolved solids and nutrients and some aquatic niches may afford an opportunity for survival of cells capable of cecal colonization. Trachoo *et al.* (13) have recently studied the survival of campylobacteria within biofilms, and such biofilms may be a more real-world approximation of the conditions encountered by campylobacteria in nature. However, we are unaware of challenge inoculation studies with campylobacteria grown in biofilms.

It is tempting to believe that VBNC cells have a purpose in the distribution and spread of campylobacteria in nature and less satisfying to view VBNC cells as merely degenerative forms, oddities, with no function. One very significant difficulty in sorting out the issue stems from the fact that presently experiments cannot be done at an earlier time point after suspension of the organism in ro-water. This is because there is a point at which three forms of the organism are coexisting—culturable spiral cells, nonculturable spiral cells, and newly formed nonculturable coccoid cells. With no mechanism for separating and sorting these, it is not technically possible to conduct meaningful *in vivo* challenge studies with such cultures. Perhaps newly transformed VBNC coccoid cells retain some ability to revert and to colonize, but presently this is mere speculation born of an intuitive notion that bacteria do not undergo such profound changes as noted here without a purpose.

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